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Isolation and Characterization of Two Binding Proteins for Advanced Glycosylation End Products from Bovine Lung Which Are Present on the Endothelial Cell Surface*

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Nonenzymatic glycosylation of proteins, as occurs at an accelerated rate in diabetes, can lead to the formation of advanced glycosylation end products of proteins (AGEs), which can bind to endothelial cells, thereby altering cellular function in a manner which could contribute to the pathogenesis of diabetic angiopathy. In this report, we describe the isolation of two endothelial cell surface-associated proteins which mediate, at least in part, the interaction of AGEs with endothelium. Based on pilot studies demonstrating AGE binding activity with comparable characteristics in bovine endothelial cell and lung extracts, the material from lung was sequentially subjected to chromatography on hydroxylapatite, fast protein liquid chromatography Mono S, and gel filtration. Two distinct polypeptides, ≈35 and ≈80 kDa, were purified to homogeneity, each of which bound AGEs as demonstrated by competitive binding assays using cellular binding proteins immobilized on a plastic surface. NH_2-terminal sequence analysis indicated that the ≈35-kDa protein was novel, whereas the NH_2-terminal sequence of the ≈80-kDa protein was identical to that of lactoferrin. Immunocytologic studies using polyclonal antibody prepared to each of the purified polypeptides demonstrated the presence of immunoreactive material on the surface of bovine endothelial cells maintained under serum-free conditions. Furthermore, immunoelectron microscopic studies with antibodies to the ≈35- and ≈80-kDa AGE-binding proteins conjugated to different size colloidal gold particles confirmed the presence of the target antigens on the cell surface and suggested that they were closely associated. IgG purified from polyclonal antisera to either the 35- or 80-kDa AGE-binding proteins blocked the binding of ^{125}I-AGE-albumin to the cell surface. These results indicate that endothelial cells express specific cell surface molecules which mediate AGE-endothelial interaction. These polypeptides represent a novel class of cell surface acceptor molecules for glucose-modified proteins which may promote degradation and/or transcytosis of the ligand, and modulation of cellular function.

Interaction of aldoses with proteins initiates a chain of nonenzymatic reactions leading to the covalent addition of advanced glycosylation end products (AGEs)[1] to proteins. AGEs are heterogeneous in structure, exhibit characteristic yellow-brown pigmentation, fluorescence, and have a propensity to form cross-links (1–3). AGEs are specifically recognized by cellular binding sites (1, 4). Because AGEs accumulate in increased amounts in hyperglycemia, and can act as toxic agents that contribute to tissue lesions of diabetes, their interaction with and processing by relevant cell types is of special interest (1). AGEs accumulate especially in vascular walls, at an accelerated rate in diabetes, and, to some extent in normal aging, where they are associated with microvascular lesions.

In previous studies, attention has been focused on monocyte-AGE interaction (1, 5) and the potential role of the monocyte as a scavenger which degrades AGEs. Recently, evidence has been provided that AGEs bind specifically to cultured endothelial cells and that AGE-endothelial interaction can result in endocytosis of AGEs, alterations in endothelial cell growth, coagulant and barrier functions, and in expression of endothelial-derived relaxing activity (4, 6–8). Moreover, AGEs have been found in the basement membranes to which endothelial cells adhere (1–3). These considerations led us to undertake the characterization and isolation of endothelial cell surface-binding proteins which comprise the putative receptor for AGE-modified proteins. We have identified two endothelial cell surface-associated proteins, an apparently unique ≈35-kDa polypeptide and a polypeptide with NH_2-terminal sequence identity to lactoferrin (termed lactoferrin-like polypeptide). We suggest that these binding proteins have a central role in mediating the interaction of this class of glucose-modified proteins with the vessel wall.

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[1] The abbreviations used are: AGEs, advanced glycosylation end products; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; LDL, low density lipoprotein; HEPES, 4-(-hydroxyethyl)-1-piperazineethanesulfonic acid; PVC, polyvinylchloride; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Reagents and Materials—AGE-albumin was prepared by incubating bovine serum albumin (BSA, fraction V; Sigma) with 250 mM glucose 6-phosphate at 37 °C for about 4 weeks in calcium-magnesium-free phosphate-buffered saline containing protease inhibitors (pepstatin A, 0.1 μg/ml, leupeptin, 0.5 μg/ml, aprotonin, 2 μg/ml, PMSF, 1.5 mM), EDTA, 1 mM, and sodium azide, 1 mM. Prothrombin, purified as described previously (9), was glycated by the identical protocol. Control proteins were exposed to 37 °C for the same time interval and in the same buffer, except that glucose 6-phosphate was omitted. The glycated proteins were yellow-brown in color, fluorescent (1), and bound to cultured endothelial cells in a manner comparable to AGE-albumin preparations employed previously (4, 10). The latter AGE-albumin preparations were used during the early phases of this work. AGE-albumin was radiolabeled by the lactoperoxidase method (11) using Enzymobeads (Bio-Rad). The final specific radioactivity of AGE-albumin was ≈1.5×10^3 cpm/ng of protein.

Human LDL and oxidized LDL were generously provided by Drs. Deckelbaum and Tabas, respectively (Columbia). Formaldehyde-modified albumin was prepared according to Horiuchi *et al.* (12). Mannan and lactoferrin were obtained from Sigma. Lactoferrin was also purified from non-lactating bovine mammary secretion and used to develop rabbit anti-bovine lactoferrin antibodies as described previously (13). Rabbit antibody to lactoferrin was used to develop an enzyme-linked immunosorbant assay for measuring lactoferrin in fetal bovine serum: undiluted samples contained about 200 ng/ml of lactoferrin.

Bovine aortic endothelial cells, from aortas of newborn calves, were grown and characterized as described previously (14), and bovine adrenal capillary endothelial cells were generously provided by Dr. M. Furie (Dept. of Pathology, State University of New York, Stonybrook). Where indicated, confluent endothelial cells were maintained for 4 days in serum-free medium containing RPMI 1640 (aortic endothelial cells)/minimal essential medium α (capillary endothelial cells) and bovine serum albumin (1%). Acetone powder of bovine lung tissue (obtained from Sigma) was used as an additional source of starting material for large scale preparations of AGE-binding proteins.

Cell Binding Studies—Binding of ^{125}I-AGE-albumin to endothelium was studied using confluent endothelial monolayers (0.32 cm^2/well) in minimal essential medium containing 1% fetal calf serum (binding buffer). In brief, wells were incubated with the above binding buffer (50 μl/well) containing ^{125}I-AGE-albumin alone (total binding) or in the presence of a 20-fold molar excess of unlabeled AGE-albumin (nonspecific binding). Comparable binding of ^{125}I-AGE-albumin was observed when binding buffer was replaced with a serum-free buffered-salt solution (HEPES 10 mM, pH 7.45, NaCl, 137 mM; glucose, 11 mM; KCl, 4 mM; $CaCl_2$ 5 mM) containing BSA (1 mg/ml). Following a 3-h incubation at 4 °C, wells were washed 10 times rapidly in binding buffer, and cell-bound ^{125}I-AGE-protein was eluted with heparin-containing buffer (HEPES 10 mM, pH 7.45; NaCl, 137 mM; glucose, 11 mM; KCl, 4 mM; EDTA, 5 mM; BSA, 1 mg/ml; and heparin, 1 mg/ml). These methods have been described in detail (4). Where indicated, endothelial cell monolayers were preincubated with antibody to purified AGE-binding proteins (2 h at 4 °C), excess unlabeled AGE-albumin or trypsin (0.05 unit/ml) for 30 min at 37 °C. The trypsin solution was aspirated, and residual trypsin was neutralized and removed during 10 washes over 10 min with the serum-containing binding buffer prior to the addition of tracer. The endothelial monolayer remained intact after exposure to trypsin under these conditions based on continued adherence of cells to the growth surface and on the ability of the cells to exclude trypan blue. In certain experiments, endothelial cells were removed from the dish surface by incubation with Tris 20 mM, pH 7.4, NaCl (0.1 M), octyl-β-glucoside (1%), PMSF (1 mM), and trasylol (0.1%), in a final volume of 0.1 ml, for 30 min at 37 °C. The dish was washed three times with binding buffer, and a radioligand binding assay was carried out using ^{125}I-AGE-albumin as described for intact endothelial cell monolayers above. In other experiments endothelial monolayers were pretreated with high salt, EDTA, acid, or phosphatidylinositol-specific phospholipase C purified from *Staphylococcus aureus* (15) (generously provided by Dr. M. Low of this department), and then a binding assay with ^{125}I-AGE-albumin was performed as described above.

Assays for Binding ^{125}I-AGE-albumin to Binding Proteins Immobilized on Polyvinylchloride Wells—These AGE-albumin binding assays were performed with endothelial cell and lung extracts, purified AGE-binding proteins, and purified lactoferrin. Samples of bovine lung extract were prepared as described below. The endothelial extract was prepared by scraping 10^9 aortic endothelial cells into suspension and concentrating the protein by acetone precipitation. The precipitate was solubilized in 12 ml of buffer, final pH 7.4, containing Tris (20 mM), NaCl (0.1 M), octyl-β-glucoside (1%), 0.1% trasylol, and PMSF (1 mM), for 4 h at 4 °C with constant agitation, centrifuged at 4 °C for 30 min at 11,000 × *g*, and the supernatant was filtered (0.45 μm). The latter material was used in a binding assay, in which putative AGE-binding proteins were allowed to adhere to wells of a polyvinylchloride plate (PVC assay). Where indicated, crude lung or endothelial cell extract, prepared as above except without protease inhibitors, was incubated with immobilized trypsin (10% (v/v), Sigma) for 1 h at 37 °C. Samples were diluted as indicated in Tris (20 mM, pH 7.4), NaCl (0.1 M), PMSF (1 mM), trasylol (0.1%), octyl-β-glucoside (0.1%), and 0.1 ml of this material was incubated at 37 °C for 3 h in wells of 96-well PVC plates (0.1 ml). Then, the mixture was aspirated, the wells were washed three times with wash buffer containing Tris, 0.02 M, NaCl, 0.1 M, Tween 20, 0.05%, and 0.1 ml of binding buffer was added for 2 h at 37 °C. The medium was aspirated and each well was incubated with 0.05 ml of binding buffer containing ^{125}I-AGE-albumin alone or in the presence of other reagents (unlabeled AGE-albumin or AGE-prothrombin, antibodies, etc.) for 3 h at 37 °C. Binding was terminated by five rapid washes in ice-cold wash buffer (0.1 ml/wash). Bound radioactivity was eluted during a 5-min incubation at 37 °C in heparin-containing buffer as above. These are the same elution conditions used to quantitate specifically bound ^{125}I-AGE-albumin in the endothelial cell radioligand binding assay described previously (4). When PVC assays were performed with binding buffer in which fetal calf serum was replaced by buffered-salt solution containing only 0.1% bovine serum albumin comparable results were obtained. Thus, although fetal calf serum contains lactoferrin, which is, by NH_2-terminal sequence, identical to the 80-kDa AGE-binding protein (see below), at a concentration of 1% serum, as in the PVC-binding buffer (which corresponded to a lactoferrin concentration of ≈2 ng/ml), lactoferrin did not alter ^{125}I-AGE-albumin interaction with the binding proteins. For PVC assays in which larger amounts of purified lactoferrin (20–500 μg/ml) were adsorbed to wells of a PVC plate, the binding assay employed an identical procedure except that binding buffer was modified to exclude any serum.

Equilibrium binding data were analyzed according to the equation of Klotz and Hunston (16) ($B = nKA/1 + KA$ where B = specifically bound ligand (total binding, wells incubated with tracer alone, minus nonspecific binding, wells incubated with tracer in the presence of excess unlabeled material), n = sites/cell, K = the dissociation constant, and A = free ligand concentration) using nonlinear least-squares analysis (Enzfitter).

Purification of AGE-binding Proteins—Bovine lung powder (30 g, Sigma) was added to Tris (20 mM), NaCl (0.1 M), PMSF (1 mM), trasylol (0.1%), and octyl-β-glucoside (1%), pH 7.4, (total of ≈300 ml) for 16 h at 4 °C with constant mixing. Insoluble material was removed by centrifugation (11,000 × *g*) for 30 min at 4 °C), the supernatant (25.2 g) was filtered (0.45 μm), and applied to hydroxylapatite (300 ml, IBF, Savage, MD). The column, which was run at ≈1 ml/min, was washed until the absorbance at 280 nm was <0.01, and then step-eluted with buffer containing 1 M NaCl. The eluate was dialyzed at 4 °C for 18 h *versus* NaCl (50 mM), sodium phosphate (50 mM), octyl-β-glucoside (0.1%), pH 5.5, and applied to an FPLC Mono S column (HR 5/5, ≈1 ml/min) equilibrated in the same buffer. After washing, the column was eluted with a linear salt gradient (50 mM to 1 M NaCl developed over 20 ml), and fractions were assayed for binding activity using the PVC assay. The active fractions were pooled and concentrated by centrifugation on Centricon membranes (Lexington, MA; molecular weight cut-off 3,000) to a final volume of ≈250 μl and then applied to gel filtration columns. The 20- and 35-kDa AGE-binding proteins (pools II and I from Mono S, respectively) were chromatographed on FPLC Superdex 75 HR 10/30 (flow rate ≈0.5 ml/min) in Tris (20 mM), NaCl (60 mM) containing octyl-β-glucoside (0.1%), pH 7.4. The 80-kDa AGE-binding protein (pool III from Mono S) was applied to HPLC TSK 250 and TSK 125 columns (Bio-Rad) run in series (flow rate, 1 ml/min) in sodium phosphate (20 mM), sodium sulfate (0.1 M), and octyl-β-glucoside (0.1%), pH 6.8. The column was calibrated using as standards equine myoglobin (M_r 17,000), ovalbumin (M_r 44,000), IgG (M_r 158,000), and thyroglobulin (M_r 670,000) (Bio-Rad).

To facilitate sequence analysis, the purified proteins from gel filtration were separately chromatographed on HPLC-reversed phase: samples were diluted 1:4 in trifluoroacetic acid (0.1%, pH 2.3), and

applied to a C_4-reversed phase column (Vydac, NJ) run at 0.2 ml/min. The column was equilibrated with trifluoroacetic acid (0.1%) and acetonitrile (10%), washed with the same buffer after sample loading, and eluted with an ascending acetonitrile gradient (10–100%). In each case, a single major peak was observed: the 35-, 20-, and 80-kDa AGE-binding proteins eluted, respectively, at acetonitrile concentrations of 34, 37, and 43%.

Purified AGE-binding proteins were analyzed by reduced and nonreduced SDS-PAGE (17) using the Phast gel system (Pharmacia LKB Biotechnology Inc., 10–15% gradient gels), and proteins in the gels were visualized with Coomassie Blue. Molecular masses were estimated from semilogarithmic plots constructed from the migration of standard proteins (myosin, 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin 69 kDa; ovalbumin 46 kDa; carbonic anhydrase 30 kDa; trypsin inhibitor 21.5 kDa; and lysozyme 14.3 kDa) (Amersham Corp.) run simultaneously.

Sequence analyses were performed with an Applied Biosystems gas-phase sequencer (model 470A, Foster City, CA). Phenylthiohydantoin amino acid derivatives were identified "on-line" with an ABI model 120 phenylthiohydantoin analyzer. NH_2-terminal sequence was entered into the program WordSearch from the sequence analysis software package by the Genetics Computer Group (18) to search the National Biomedical Research Foundation protein database.

The AGE binding activity of the purified binding proteins was studied using AGE-albumin Affi-Gel, in addition to studies employing the PVC assay. AGE-albumin was coupled to Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions. The concentration of ligand bound was ≈5 mg/ml of gel. Control albumin preparations (incubated at 37 °C under the same conditions except no glucose 6-phosphate was present) were coupled to Affi-Gel 15 in an identical manner. Purified binding proteins from the gel filtration column (≈0.1 mg/ml, 1 ml) were chromatographed over a 5-ml AGE-albumin column previously equilibrated with Tris (20 mM), NaCl (0.1 M), and octyl-β-glucoside (0.1%), pH 7.4. The column was then washed with 10 bed volumes of the same buffer and eluted with 10 ml of Tris (20 mM), NaCl (1 M), and 0.1% octyl-β-glucoside, pH 7.4.

Production of Antisera to AGE-binding Proteins—For the production of antisera to each of the AGE-binding proteins, we employed electrophoretically homogeneous preparations obtained after the gel filtration step. Guinea pigs were immunized by standard methods (19), IgG from the sera was purified by chromatography on FPLC protein A-Superose. Adsorbed IgG was eluted with acetic acid (0.58%), NaCl (0.1 M), the pH of the eluate was brought to ≈7.5 with sodium bicarbonate, and immunoglobulins were dialyzed *versus* Tris (20 mM)-buffered saline (0.1 M, final pH 7.4).

Immunoblotting—Samples were prepared from cultured aortic and capillary endothelial cells by the following procedure: endothelial cell pellets from ≈10^7 cells were treated with trichloroacetic acid (final concentration 20%, 5 min), washed three times with ice-cold acetone, and then resuspended in SDS-PAGE sample buffer without reducing agents (17). Purified AGE-binding proteins (see below) derived from lung extract were also used for immunoblotting. Samples in SDS-PAGE buffer were applied to 1.5-mm thick polyacrylamide gels (10% SDS-PAGE), and, after electrophoresis, proteins were transferred electrophoretically to nitrocellulose using a semi-dry blotting system for about 50 min at 130 mA. Nitrocellulose membranes with immobilized AGE-binding proteins were reacted with antisera to the purified AGE proteins or with antibody to bovine lactoferrin. For the 35-kDa AGE-binding protein antisera, the Blotto procedure, using nonfat dry milk for the blocking step, was followed (20). For the anti-80-kDa AGE-binding protein and anti-lactoferrin IgG, the blotting procedure employed Tris (20 mM, pH 7.4), NaCl (0.1 M), and bovine serum albumin (Sigma, Fraction V; 5%) for the blocking step. Immunoreactive bands were identified by the horseradish peroxidase method (according to a kit from Amersham Corp.).

Immunofluorescence and Immunoelectron Microscopic Studies—Indirect immunofluorescence was performed on nonpermeabilized endothelial cell cultures which had been maintained in serum-free medium (minimal essential medium containing albumin (1%), high density lipoprotein (10 μg/ml), transferrin (5 μg/ml), and fibroblast growth factor (3 ng/ml)). Monolayers of bovine endothelial cells on coverslips were fixed using paraformaldehyde (2%) in phosphate-buffered saline, pH 7.2, for 5–10 min and then washed in the same buffer. The distribution of the 35- and 80-kDa AGE-binding proteins was determined by indirect immunofluorescence using purified IgG from guinea pigs immunized with these binding proteins or nonimmune sera, by the procedure previously employed for visualizing endothelial cell associated ligands (14, 21, 22). Immune IgG to the 35- and 80-kDa AGE-binding proteins, as well as nonimmune guinea pig IgG, was conjugated to gold particles (21), reacted with the endothelium at 4 °C, and then prepared for electron microscopy, as described (22).

RESULTS

Characterization of AGE Binding Activity in Extracts from Bovine Aortic Endothelial Cells and Bovine Lung Tissue

AGE-albumin binds selectively to the surface of bovine aortic endothelial cells (Fig. 1 and Ref. 4). Inhibition of ^{125}I-

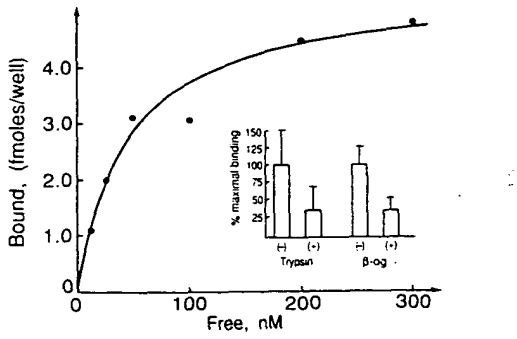

FIG. 1. **Binding of ^{125}I-AGE-albumin to bovine aortic endothelium: effects of trypsin and detergent.** Confluent endothelial monolayers were incubated with the indicated concentrations of ^{125}I-AGE-albumin alone (total binding) or in the presence of a 20-fold molar excess of unlabeled AGE-albumin (nonspecific), and a radioligand binding assay was performed. Specific binding (the difference of total and nonspecific binding) is shown. Data were analyzed by the nonlinear least-squares program, and the curve indicates the best fit line. Parameters of binding fit to a one-site model: $K_d = 43 \pm 8$ nM and $n = 5.4 \pm 0.3$ fmol/well. The *inset* shows the effect of pretreatment of endothelial monolayers with trypsin or detergent (octyl-β-glucoside, 1%) on the subsequent binding of ^{125}I-AGE-albumin (100 nM) in the presence/absence of a 20-fold molar excess of unlabeled AGE-albumin. Data are shown as percent maximal specific binding, and 100% binding is defined as that observed in the absence of a specific pretreatment (*i.e.* buffer alone designated (−)). Samples run with the indicated pretreatment are designated by (+).

TABLE I

Treatment of endothelial cultures with high salt, EDTA, acid or phospholipase C did not affect binding of ^{125}I-AGE-albumin

Confluent endothelial monolayers were incubated either with serum-free minimal essential medium, NaCl (1 or 0.5 M), EDTA (5 mM) or acetate (0.05 M, pH 3 or 7) containing 0.15 M NaCl for 8 min at 4 °C. In other experiments purified phosphatidylinositol-specific phospholipase C (1/1 = 850 units/ml) was incubated with endothelial cells in minimal essential medium (serum-free) for 1 h at 37 °C. Cultures were washed free of the agent used in the pretreatment, and then a radioligand binding assay was performed with ^{125}I-AGE-albumin (100 nM) alone or in the presence of 20-fold molar excess of unlabeled AGE albumin as described in the text. Maximal specific binding, observed when monolayers were pretreated with minimal essential medium alone (6.7 fmol/well), was defined as 100%. The mean of duplicate determinations of specific binding (total minus nonspecific binding) is shown.

Pretreatment	% Maximal binding
NaCl 1.0 M	167
NaCl 0.5 M	108
EDTA 5 mM	107
pH 3	126
pH 7	96
Phosphatidylinositol-specific phospholipase C	
1:100	95
1:1000	90
1:10,000	93

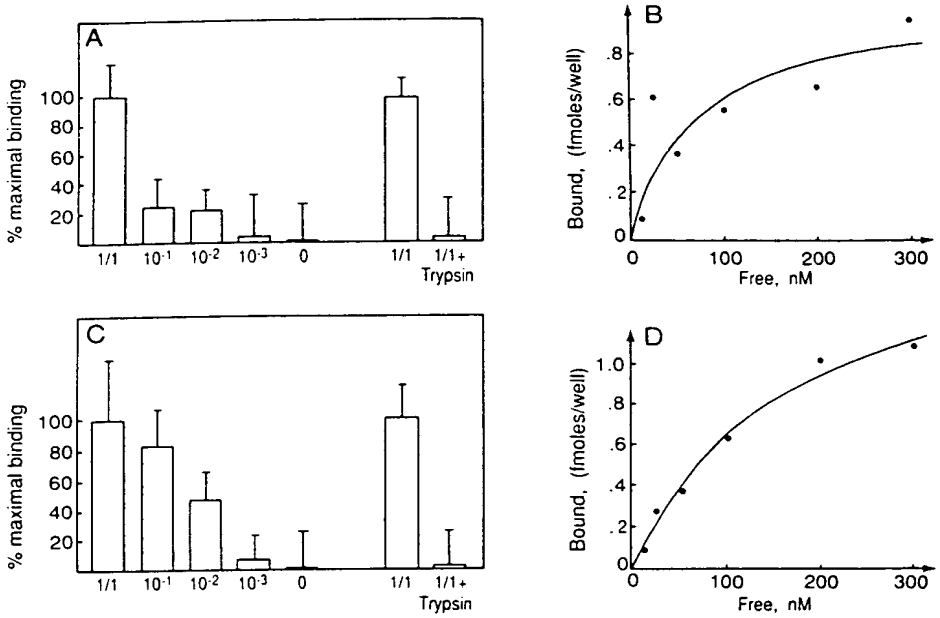

FIG. 2. **An assay for solubilized AGE-binding sites using aortic endothelial cell (*A* and *B*) and lung extracts (*C* and *D*).** *Panel A*, endothelial cells (10^9 cells) were extracted with octyl-β-glucoside-containing buffer as described in the text, diluted as indicated, and incubated in PVC wells. The PVC radioligand binding assay with ^{125}I-AGE-albumin was carried out by adding tracer alone (90 nM) or in the presence of a 20-fold molar excess of unlabeled AGE-albumin. Specific binding is shown as a % of maximal binding (100% was arbitrarily defined as the specific binding observed with undiluted extract) and is plotted *versus* extract dilution. The *right hand portion* of *panel A* shows the effect of pretreatment of the undiluted extract with trypsin. The mean ± S.E. from triplicate determinations is shown. *Panel B*, ^{125}I-AGE-albumin binding to immobilized endothelial extract. Endothelial extracts (undiluted) were adsorbed to wells of a PVC plate and then a radioligand binding assay was carried out by adding varying concentrations of ^{125}I-AGE-albumin alone or in the presence of a 20-fold excess of unlabeled AGE-albumin. Specific binding is plotted *versus* free ^{125}I-AGE-BSA. Data were analyzed by the nonlinear least-squares program, and the curve indicates the best fit line. Parameters of binding were $K_d = 70 \pm 7$ nM and ≈1.0 ± 0.03 fmol bound/well at saturation. *Panel C*, PVC plates were incubated with extracts from bovine lung at the indicated dilutions, and the binding assay was carried out as described in *panel A* above. 100% binding was defined arbitrarily as that observed in the presence of undiluted lung extract (as above). Trypsin pretreatment was carried out as in *panel A*. The mean ± S.E. from triplicate determinations is shown. *Panel D*, wells of a PVC plate were incubated with undiluted lung extract, and a radioligand binding assay was carried out as in *panel B* above. Parameters of binding were $K_d = 149 \pm 31$ nM and 1.7 ± 0.17 fmol bound/well at saturation.

AGE-albumin-endothelial cell interaction by excess unlabeled AGE-albumin and other AGE-modified proteins, but not their native forms or the components of fetal calf serum, indicated that the cellular binding site recognized the AGE-modified form of the protein rather than a determinant on albumin (control proteins were incubated under the same conditions employed for glycosylation except that glucose 6-phosphate was omitted from the reaction mixture) (4). When aortic endothelial cells were treated with detergent to elute membrane proteins, specific binding of ^{125}I-AGE-albumin to the endothelial cell skeletons was largely prevented (Fig. 1, *inset*). Pretreatment of endothelial monolayers with trypsin also blocked subsequent ^{125}I-AGE-albumin binding (Fig. 1, *inset*). Similar results were observed with cultured bovine microvascular endothelial cells (data not shown). The endothelial cell-binding sites for AGE-albumin appeared to be closely associated with the cell surface as they were not eluted by pretreatment of cultures with high salt (NaCl, 1 M), EDTA (5 mM), acid, pH 3, or phosphatidylinositol-specific phospholipase C (Table I). The latter enzyme releases proteins that are associated with the cell surface via a glycosyl-phosphatidylinositol anchor (23). Under the conditions employed here, a glycosyl-phosphoinositol anchored protein, such as Thy-1 on the surface of lymphocytes would have been removed (23).

Proteins extracted with detergent from endothelial cells and immobilized on the surface of PVC wells bound AGEs (Fig. 2). Binding of ^{125}I-AGE-albumin was dependent on the extract concentration, was half-maximal at an AGE-albumin concentration of ≈70 nM, and could be prevented by preincubation of the extract with trypsin (Fig. 2, *A* and *B*). Taken together, these data suggested that endothelial binding of AGE-albumin was likely to be due to a cell surface, detergent-extractable, trypsin-sensitive polypeptide which, after extraction from the cell surface by solubilization with octyl-β-glucoside, retained the ability to bind AGEs in a manner comparable to that observed on intact endothelial monolayers. Furthermore, other preliminary studies demonstrated that ligands-competitors for previously characterized scavenger or fucose-mannose receptors (24–28), such as oxidized LDL, acetylated LDL, mannan, and formaldehyde-modified albumin, did not block the binding of ^{125}I-AGE-albumin to endothelial cells (data not shown), raising the possibility that the cellular binding site for AGEs might be a unique receptor.

Since cultured endothelial cells are a source of only limited amounts of starting material for purification of the cell surface receptor, we turned to an endothelial cell-rich tissue, the lung. Extracts of bovine lung solubilized in the same buffer used for the endothelial cell experiments demonstrated similar properties for the binding of ^{125}I-AGE-albumin in the PVC assay. Following adsorption of lung extract to PVC wells, the binding of ^{125}I-AGE-albumin was dependent on the extract concentration and was blocked by preincubation of the extract with trypsin (Fig. 2*C*). In addition, binding of the ligand was half-maximal at an ^{125}I-AGE-albumin concentration of ≈150

nM (Fig. 2*D*), which is similar, although not identical, to that observed with endothelial cell extracts (Fig. 2*B*). Thus, lung extract, although more heterogeneous than that derived from cultured endothelial cells, appeared to be a reasonable starting material for purification of putative AGE-binding proteins with similar properties to those derived from cultured endothelium.

Purification of AGE-binding Proteins

Lung extract was chromatographed on an hydroxylapatite column, and the resin was washed extensively to remove nonadherent material, which constituted ≈99% of the applied protein (Fig. 3*A* and *flow chart* in Fig. 5). The elution profile, following application of buffer containing 1 M sodium chloride showed a single major peak of AGE-binding activity which coincided with the bulk of the protein in the effluent (Fig. 3*A*). Chromatography of the active pool from hydroxylapatite on FPLC Mono S (Fig. 3*B*) resolved three major peaks of AGE binding activity eluting at ≈0.36 M NaCl (*pool II*), ≈0.6 M NaCl (*pool I*), and ≈0.75 M NaCl (*pool III*). The fractions containing maximal activity from each of these peaks from the Mono S column were pooled separately and subjected to gel filtration on FPLC. Mono S pool I eluted from gel filtration as a single protein peak, comigrating with the AGE binding activity, and corresponding to the elution volume of a protein of ≈35 kDa (Fig. 4*A*). SDS-PAGE demonstrated a single band of≈ 35 kDa under reducing and nonreducing conditions (Fig. 4*A*, *inset*). The pool from Mono S eluting at 0.36 M NaCl (*pool II*) had two major protein peaks in its elution profile: the earlier peak coeluted with the ≈35-kDa AGE-binding protein and the later peak corresponded to the elution volume of a protein of ≈ 15 kDa (Fig 4*B*). SDS-PAGE of the material in the later eluting peak demonstrated a single band at molecular mass corresponding to ≈20 kDa under reducing and nonreducing conditions (Fig 4*B*, *inset*). The pool of AGE binding activity eluting with the highest salt concentration from Mono S (*pool III*) demonstrated a single peak on gel filtration which corresponded to the elution volume of a protein of ≈ 86 kDa (Fig. 4*C*). On SDS-PAGE this material migrated as a single band, with a molecular mass of ≈ 80 kDa, both under reduced and nonreduced conditions (Fig. 4*C*, *inset*).

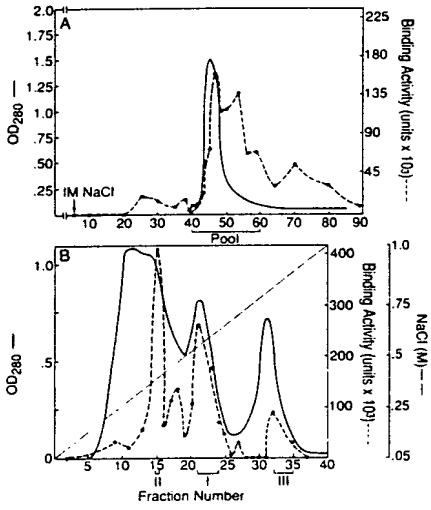

FIG. 3. **Purification of AGE-binding proteins.** *Panel A*, detergent extract of bovine lung (30 g) was applied to a hydroxylapatite column, the column was washed in equilibration buffer, and eluted with 1 M NaCl. OD_{280} (*solid line*) and binding activity in the PVC assay (*broken line*) (assayed at 1:1 dilution with ^{125}I-AGE-albumin at 100 nM) are plotted for each fraction. One binding unit is defined as one count/minute of specific binding of ^{125}I-AGE-albumin in the PVC assay/milliliter of sample applied to a PVC plate at a 1:1 dilution. The active pool of material applied to Mono S included fractions 41–60, and is indicated. *Panel B*, FPLC Mono S. The pool with AGE binding activity from the hydroxylapatite column was dialyzed and applied to FPLC Mono S. The column was washed with equilibration buffer and eluted with an ascending salt gradient (50 mM to 1 M). OD_{280}, salt concentration of the gradient, and binding units in the PVC assay are plotted for each fraction. Pools of fractions from Mono S subsequently applied to FPLC gel filtration are arbitrarily *labeled I, II,* and *III*.

A summary of our preparation of purified AGE-binding proteins is shown in Fig. 5. Starting with 30 g of lung powder, a total of ≈0.82, 0.79, and 1.1 mg of purified 35-, 20-, and 80-kDa AGE-albumin-binding proteins were obtained (note that four, one, and five runs of the gel filtration column were required to fully process all the material from one preparation of the 35-, 20-, and 80-kDa binding proteins, respectively).

Characterization of AGE-binding Proteins

Purified AGE-binding proteins were further characterized using four types of assays: first for their ability to bind AGEs by affinity chromatography on immobilized AGE-albumin columns; second for AGE binding capacity using the PVC assay; third by amino acid sequencing; and, fourth using antibodies raised to each of the AGE-binding proteins.

Affinity Chromatography on AGE-Albumin Columns—The purified AGE-binding proteins were incubated with AGE-

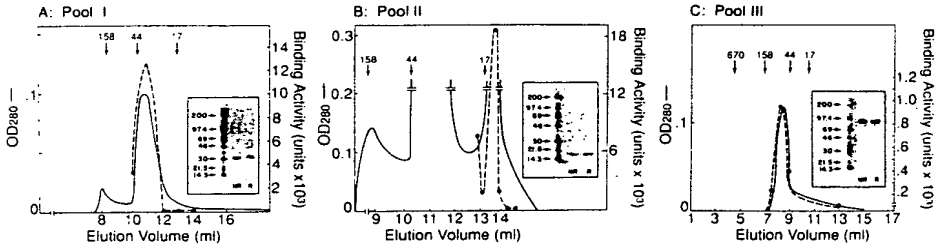

FIG. 4. **Gel filtration of Mono S AGE binding pools.** Pools I, II, and III from the FPLC Mono S column were subjected separately to gel filtration as described in the text. The elution profile is shown for pools I (*A*, 35-kDa AGE-binding protein), II (*B*, 20-kDa AGE-binding protein), and III (*C*, 80-kDa AGE-binding protein). For pools I and II an FPLC Superdex 75 column was employed and for pool III HPLC TSK 250 and 125 columns in series were used. Binding units of ^{125}I-AGE-albumin in the PVC assay are expressed as described in the legend to Fig. 3. The purified ≈20-kDa AGE-binding protein (*pool II, B*) was in the last peak eluted from the column. The material in this peak was pooled, and analyzed by SDS-PAGE (as shown in the *inset*). *Insets*: SDS-PAGE (*Phast gels*) stained with Coomassie Blue of the pooled material eluted from the gel filtration columns with peak ^{125}I-AGE-albumin binding activity. In each case, the sample contained ≈0.6 μg of protein. *NR*, nonreduced; *R*, reduced. Migration of molecular weight markers is indicated by *arrows* (*numbers* indicate molecular masses in kDa).

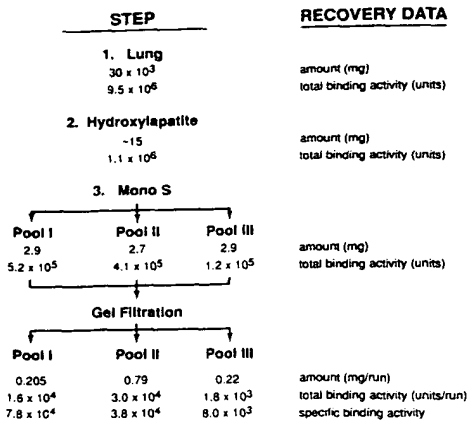

FIG. 5. **Flow diagram of preparation and recovery of AGE-binding proteins from lung extract.** For each step in the preparation, the total amount of protein and total binding activity in the PVC assay (the latter is specifically bound ^{125}I-AGE-albumin in the presence of a 1:1 dilution of sample multiplied by the volume at that point in the preparation) is shown. Note that the gel filtration column was run four times and once, respectively, to fully process the material in pools I and II from Mono S. The HPLC gel filtration column was run five times to fully process the material in pool III from Mono S. The specific binding activity (total binding activity divided by the number of milligrams of protein at that step) is shown for the preparations after gel filtration, when the three AGE-binding proteins were separated from each other (for the gel filtration step, the specific binding activity is expressed as binding units/mg protein for one representative run of the gel filtration column).

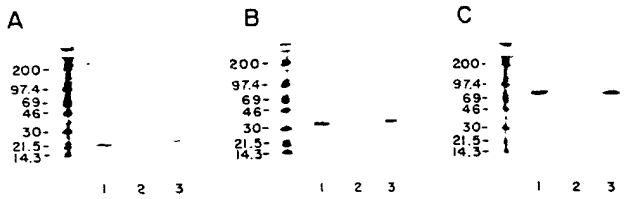

FIG. 6. **Adsorption of purified AGE-binding proteins to AGE-albumin Affi-Gel.** Each of the purified AGE-binding proteins, 20 kDa (*A*), 35 kDa (*B*), 80 kDa (*C*) (100 μg of each) was dialyzed *versus* Tris-buffered saline containing 0.1% octyl-β-glucoside (1 liter, 16 h, 4 °C), and chromatographed on AGE-albumin Affi-Gel equilibrated in the same buffer. The column was washed, and then eluted with 1 M sodium chloride. SDS-PAGE of the initial sample (*1*), the wash after adsorption of the sample to the AGE-albumin Affi-Gel column (*2*) (in this case, samples were concentrated to a comparable volume to that for *samples 1* and *3*), and the 1 M NaCl eluate of the column (*3*) is shown in each case. Samples from the columns were dialyzed *versus* Tris-buffered saline, boiled in SDS-gel sample buffer, and subjected to SDS-PAGE (*Phast gels* 10–15% gradient). The gel bands, visualized by Coomassie Blue staining, correspond to ≈20 kDa (*panel A*, in the first and third lanes about 0.3 and 0.2 μg of protein were loaded, respectively, and in the second lane a comparable volume of sample was loaded), ≈35 kDa (*panel B*, protein content of samples was as in *panel A*), and ≈80 kDa (*panel C*, about 1 and 0.8 μg of protein were added in both the *first* and *third lanes* of the gel, respectively, and in the *second lane* a comparable volume of sample was loaded). Molecular weights were estimated based on the migration of standards run simultaneously (*arrows* indicate migration of 200, 97.4, 69, 46, 30, 21.5, and 14.3 kDa standards).

albumin immobilized on Affi-Gel. Each binding protein adsorbed to the resin and could be eluted with 1 M NaCl (Fig. 6, *A-C*). In contrast, native albumin linked to Affi-Gel did not adsorb the binding proteins (data not shown).

PVC Assay of AGE-binding Proteins—Purified AGE-binding proteins immobilized on wells of PVC plates also bound ^{125}I-AGE-albumin. Specific binding was proportional to the amount of binding protein adsorbed to the plastic surface (Fig. 7, *AI*, *BI*, and *CI*), depended on the concentration of ^{125}I-AGE-albumin (Fig. 7, *AII*, *BII*, and *CII*), and could be largely blocked by excess unlabeled AGE-albumin or AGE-prothrombin (Fig. 7, *AIII*, *BIII*, and *CIII*). Neither native albumin (data not shown) nor prothrombin had any significant effect on the binding of ^{125}I-AGE-albumin to the purified binding proteins. Binding was half-maximal at ^{125}I-AGE-albumin concentrations of ≈60, ≈43, and ≈240 nM with 35-, 20-, or 80-kDa AGE-binding protein immobilized on the plastic surface, respectively (Fig. 7, *AII*, *BII*, and *CII*).

NH_2-terminal Sequence Analysis and Amino Acid Analysis of AGE-binding Proteins—To further characterize the purified AGE-binding proteins, NH_2-terminal sequencing (Table II) was performed and compared with sequences currently reported in the database (National Biomedical Research Foundation protein data bases). No known sequences were found which matched closely that of the 35-kDa binding protein (Table II, section A). In contrast, there was identity between the amino-terminal sequence of the 20-kDa AGE-binding protein and that previously reported for bovine high mobility 1 protein (29) (Table II, section B; 24 out of 25 residues), one of the most abundant members of the high mobility group of non-histone chromosomal proteins (30). The sequence of the amino-terminal 80-kDa AGE-binding protein displayed virtual identity to the amino-terminal sequence of bovine lactoferrin (13) (Table II, section C), leading us to tentatively assign it the name lactoferrin-like AGE-binding protein.

Immunologic Analysis of AGE-binding Proteins—In view of the identification of the 20-kDa AGE-binding protein as a nuclear-associated protein which was not likely to function as a cell surface receptor, our attention was focused on the 35- and 80-kDa AGE-binding proteins. Immunoblotting was performed employing IgG from animals immunized with the two purified proteins. In each case, the polyclonal antibody allowed visualization of the immunogen on Western blots (Fig. 8, *A-B*) but did not detect the other AGE-binding protein. Appearance of these bands was blocked by the addition of the corresponding purified soluble AGE-binding protein during incubation of Western blots with the primary antibody (data not shown). In contrast, addition of the other soluble purified AGE-binding protein was without effect. Consistent with the apparent specificity of the antibodies for the AGE-binding protein used as the immunogen, each antibody largely blocked binding of ^{125}I-AGE-albumin to the respective AGE-binding protein in a dose-dependent manner, but not to the other binding proteins (Fig. 7, *A-C*, *panel III*).

In view of the NH_2-terminal sequence identity between the 80-kDa AGE-binding protein and lactoferrin, further studies were performed with anti-lactoferrin antibody and lactoferrin purified to homogeneity from non-lactating bovine mammary secretion. On nonreduced SDS-PAGE, lactoferrin and the 80-kDa AGE-binding protein comigrated (Fig. 9*A*). Immunoblotting demonstrated that anti-lactoferrin antibody bound to the 80-kDa AGE-binding protein and lactoferrin (Fig. 9*B*). Similarly, immunoblotting studies demonstrated that the anti-80-kDa AGE-binding protein antibodies recognized both the 80-kDa AGE-binding protein and lactoferrin (Fig. 8*C*). These results led us to study whether lactoferrin bound ^{125}I-AGE albumin in the PVC assay. The binding of ^{125}I-AGE albumin to lactoferrin immobilized on the plastic surface depended on the amount of lactoferrin used to coat the wells (Fig. 9*C*), and, at a constant lactoferrin concentration, was half-maximal at [^{125}I-AGE-albumin] ≈270 nM (Fig. 9*D*). This

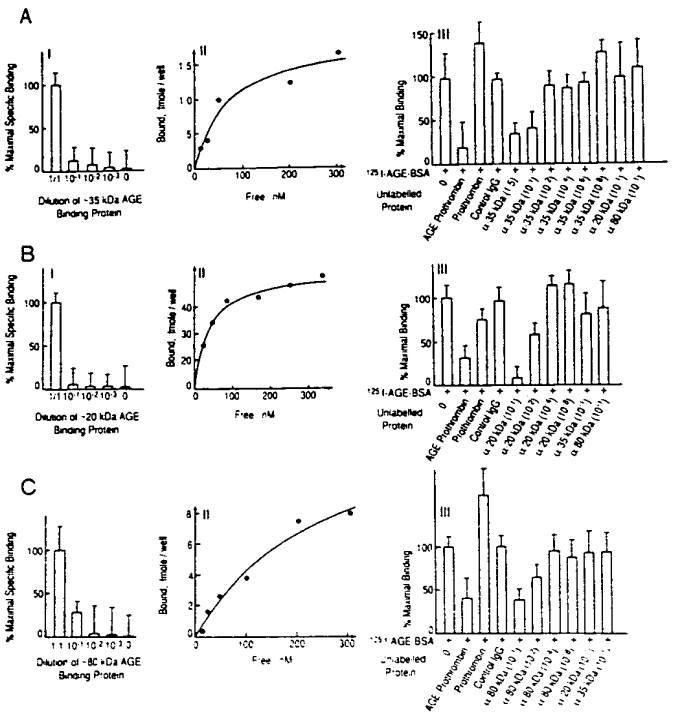

FIG. 7. **Interaction of purified AGE-binding proteins with AGE-modified proteins and immune IgGs raised against the ≈35-, ≈20-, and ≈80-kDa AGE-binding protein.** Wells were coated with purified AGE-binding proteins (*A*, 35 kDa; *B*, 20 kDa; *C*, 80 kDa) and then PVC radioligand binding assays were performed with ^{125}I-AGE-albumin as indicated. *Panel AI*, wells were coated with the indicated dilutions of purified ≈35-kDa AGE-binding protein (1:1 = undiluted ≈200 μg/ml), and then incubated with minimal essential medium containing 1% fetal bovine serum and ^{125}I-AGE-albumin (90 nM) alone or in the presence of a 20-fold excess of unlabeled AGE-albumin. Specific binding is shown (mean ± S.E. of triplicate determinations). Maximal specific binding of ligand (34 fmol/well) was defined arbitrarily as 100% in wells with undiluted binding protein. *Panel AII*, wells were coated with the ≈35-kDa AGE-binding protein (10 μg/well) and a radioligand binding assay was performed by adding varying concentrations of ^{125}I-AGE-albumin alone or in the presence of a 20-fold molar excess of unlabeled AGE-albumin. Specific binding is plotted *versus* free ^{125}I-AGE-albumin. Data were analyzed by the nonlinear least-squares program, and the curve indicates the best fit line. Parameters of binding were K_d = 61 ± 23 nM and 1.85 ± 0.25 fmol bound/well at saturation. *Panel AIII*, wells were coated with ≈35-kDa AGE-binding protein (10 μg/well), and then a radioligand binding assay was carried out with ^{125}I-AGE-albumin alone (90 nM) (arbitrarily defined as 100%) or in the presence of the indicated unlabeled protein: AGE-albumin (2 μM), AGE-prothrombin (2 μM), or native prothrombin (3 μM). In other experiments, after coating wells with ≈35-kDa AGE-binding protein and blocking excess sites on the plastic wells with fetal bovine serum, wells were incubated with the indicated IgG for 1 h at 37 °C prior to the binding assay: control or nonimmune IgG (10 μg/ml), anti-35-kDa AGE-binding protein IgG (α-35 kDa; 1:10 dilution = 100 μg/ml, other dilutions as indicated), anti-20-kDa AGE-binding protein IgG (α-20 kDa; 1:10 = 100 μg/ml) or anti-80-kDa AGE-binding protein IgG (α-80 kDa; 1:10 = 100 μg/ml). Following washing to remove unbound antibody, a radioligand binding assay was performed with ^{125}I-AGE-albumin (90 nM). Percent maximal total binding, defined arbitrarily as 100% in the presence of ^{125}I-AGE-albumin alone, is shown (mean ± S.E. of triplicate determinations). *Panel BI*, wells were coated with the indicated dilutions of purified ≈20-kDa AGE-binding protein (1:1 dilution ≈200 μg/ml), and a radioligand binding assay was performed as in *panel AI*. Percent maximal specific binding of ligand (38 fmol/well) was defined as 100% in wells with undiluted binding protein (as in *panel AI* above). *Panel BII*, wells were coated with undiluted ≈20-kDa AGE-binding protein (10 μg/well), and a radioligand binding assay was performed in the presence of varying concentrations of ^{125}I-AGE-albumin as in *panel AII* above. Specific binding is plotted *versus* free ^{125}I-AGE-albumin, and data were analyzed as in *panel AII* above. Parameters of binding were K_d = 43 ± 6 nM and 60 ± 2 fmol bound/well at saturation. *Panel BIII*, wells were coated with undiluted ≈20-kDa AGE-binding protein (10 μg/ml), and then a radioligand binding assay was carried out as in *panel AIII*. Experiments with anti-AGE-binding protein IgGs were also performed as in *panel AIII* above, except that dilutions of anti-20-kDa IgG were used, whereas only the 1:10 dilution of anti-35- and anti-80-kDa AGE-binding protein IgGs was employed. *Panel CI*, wells were coated with the indicated dilutions of purified ≈80-kDa AGE-binding protein (1:1 dilution ≈200 μg/ml), and then a radioligand binding assay was performed as in *panel AI* above. Percent maximal specific binding of ligand (1.2 fmol/well) was defined as 100% in wells with undiluted binding protein. *Panel CII*, wells were coated with ≈80-kDa AGE-binding protein (10 μg/well), and a radioligand binding assay in the presence of varying concentrations of ^{125}I-AGE-albumin was performed as in *panel AII* above. Specific binding is plotted *versus* free ^{125}I-AGE-albumin, and data were analyzed as in *panel AII* above. Parameters of binding were K_d = 238 ± 0.6 nM and 1.5 ± 0.01 fmol bound/well at saturation. *Panel CIII*, wells were coated with ≈80-kDa AGE-binding protein (10 μg/well), and then a radioligand binding assay was carried out as described in *panel AIII* above except that dilutions of anti-80-kDa AGE-binding protein IgG were used, whereas only the 1:10 dilution of anti-20-kDa and anti-35-kDa AGE-binding protein IgGs was employed. In each case (*A-C*), the mean of four-six replicates is shown.

is similar to the K_d for ^{125}I-AGE-albumin binding to the 80-kDa AGE-binding protein (≈240 nM).

Endothelial Association of AGE-binding Proteins—To determine whether endothelial cells express AGE-binding proteins, immunoblotting was performed on material derived from cultured bovine aortic endothelial cells (Fig. 8, *A-B*). Immunoblotting with the anti-35-kDa AGE-binding protein detected a band in endothelial cells of ≈35 kDa (Fig. 8*A*, *lane 4*). Immunoreactive material recognized in endothelial extracts by antibody raised to the 80-kDa AGE-binding protein (Fig. 8*B*, *lane 4*) and antibodies raised to lactoferrin (Fig. 9*B*, *lane 2*) migrated with a molecular mass of ≈30 kDa. This was not due to cross-reactivity with the 35-kDa AGE-binding protein (as indicated by the studies above in Figs. 8, *A-B*, and 9*B*), but possibly cleavage of the 80-kDa AGE-binding protein either on the surface of cultured endothelial cells or during our preparation of the extract. Pilot studies in which purified 80-kDa AGE-binding protein was subjected to the identical conditions did not demonstrate cleavage. Thus, it is likely that cell-dependent processes or a protease in the culture medium resulted in the observed cleavage. In this context, cleavage of lactoferrin under a variety of conditions has been observed (13, 31, 32). Alternatively, the endothelial cells may synthesize a smaller lactoferrin-like polypeptide immunoreactive with the anti-80-kDa and anti-lactoferrin antibodies. Further studies are in progress to clarify these issues.

Since endothelial cells were likely to contribute to the pool of ≈80-kDa AGE-binding protein isolated from the lung, one would have expected that our preparations would have shown evidence of the ≈30 kDa form. In fact, when crude lung extract was subjected to Western blotting, both forms were observed. Hydroxylapatite chromatography, employed as a first step in the purification procedure, separated these two forms: Western blotting showed immunoreactive protein of ≈80 kDa in the column eluate and the ≈30 kDa form to be in the fall through (data not shown). Thus, material purified by our procedure from lung consisted only of the ≈80 kDa form.

Indirect immunofluorescence of nonpermeabilized monolayers of bovine aortic endothelial cells maintained under serum-free conditions, using IgG prepared from antiserum to the 35- and 80-kDa AGE-binding proteins, displayed both in a diffusely punctate pattern, whereas nonimmune IgG showed

TABLE II

Amino-terminal sequence analysis of AGE-binding proteins (AGE-BP), bovine high mobility 1 protein (BoHMGI), and bovine lactoferrin (Bolact)

A, Ala; C, Cys; D, Asp; E, Glu; F, Phe, G, Gly, H, His, I, Ile; K, Lys; L, Leu, M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp and Y, Tyr. *X* is an amino acid residue not identified at that position.

	A
35 kDa AGE-BP:	D Q *X* I T A R I G K P L V L N *X* K G A P K K P P Q Q L *X X* K L N
	B[a]
20 kDa AGE-BP:	G K G D P K K P R G K M S S Y A F F V Q T X R E E
BoHMG1:	G K G D P K K P R G K M S S Y A F F V Q T C R E E
	C[b]
80 kDa AGE-BP:	A P R K N V R W *X* T I S Q P E W F K
Bolact:	A P R K N V R W *X* T I S Q P E W F K

[a] Alignment of 20-kDa AGE-binding protein with bovine high mobility group 1 protein as reported (29).
[b] Alignment of 80-kDa AGE-binding protein with bovine lactoferrin as reported by Rejman *et al.* (13).

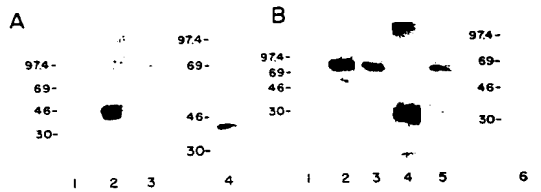

FIG. 8. **Characterization of 35- and 80-kDa AGE-binding proteins by immunoblotting (*A*, 35 kDa; *B*, 80 kDa) using immunoblotting with polyclonal immune IgG to each of the binding proteins.** Blots were analyzed by the horseradish peroxidase method. *Panel A*, immunoblotting using immune IgG raised against the 35-kDa AGE-binding protein reacted with each of the following: *lane 1*, purified 20-kDa AGE-binding protein (4.5 μg); *lane 2*, purified 35-kDa AGE-binding protein (4.5 μg); *lane 3*, purified 80-kDa AGE-binding protein (4.5 μg); and *lane 4*, endothelial cell extract (9 μg). *Panel B*, immunoblotting using the immune IgG raised against the 80-kDa AGE-binding protein reacted with each of the following: *lane 1*, purified 20-kDa AGE-binding protein (4.5 μg); *lane 2*, purified lactoferrin (7 μg); *lane 3*, commercial lactoferrin (Sigma, 7 μg); *lane 4*, endothelial cell extract (9 μg); *lane 5*, purified 80-kDa AGE-binding protein (4.5 μg); and *lane 6*, purified 35-kDa AGE-binding protein (4.5 μg). Samples were subjected to nonreduced SDS-PAGE, transferred to nitrocellulose, and incubated with the indicated immune IgG (50 μg/ml; *panel A*, anti-35-kDa AGE-binding protein, and *panel B*, anti-80-kDa AGE-binding protein). Sites of primary antibody binding were visualized with horseradish peroxidase-labeled secondary antibody. Migration of standards is indicated by the *arrows* (corresponding to molecular masses of 97.4, 69, 46, and 30 kDa). The 21.5- and 14.3-kDa standards ran very close to the 30-kDa standard on these gels, and thus are not marked separately. Migration of the 20-kDa AGE-binding protein was just below the closely spaced 21-kDa marker.

no staining (Fig. 10, *A-C*). A similar distribution of these two cell surface antigens was observed on capillary endothelial cells (Fig. 10, *D-F*). Furthermore, immunoelectron microscopic studies in which immune IgG to either the 35- or 80-kDa AGE-binding proteins was coupled to 12- or 24-nm colloidal gold particles, respectively, demonstrated close association of gold particles with the endothelial cell surface (Fig. 10, *G* and *H*) (high-power fields are shown in Fig. 10, *G-I*, to allow differentiation of 12- and 24-nm gold particles, thus only one or at most two gold particles or groups of gold particles are seen in each of the three panels). Nonimmune IgG coupled to gold particles did not demonstrate similar binding. When endothelial cells were reacted simultaneously with both antibodies, the two different size gold particles were scattered in clusters over the cell surface. Each cluster contained both sizes of gold particles (Fig. 10*I*), suggesting that the 35- and 80-kDa AGE-binding proteins were colocalized on the cell surface.

These findings suggested the possibility that polypeptides immunoreactive with the 35-kDa and lactoferrin-like AGE-binding proteins might function as the cell surface acceptor sites for ^{125}I-AGE-albumin. Radioligand binding studies, carried out after preincubation of endothelium with immune IgG to each of the binding proteins, demonstrated that specific binding was blocked by antibody to the 35- or 80-kDa polypeptide (Fig. 11, *A* and *C*). The effect of these IgGs was dependent on their concentration, and no inhibition of ^{125}I-AGE-albumin binding to endothelium was observed with nonimmune guinea pig IgG. In addition, antibody to the 20-kDa AGE-binding protein had no effect on the binding of ^{125}I-AGE-albumin to endothelium (Fig. 11*B*).

DISCUSSION

In previous work we showed that ^{125}I-AGE-albumin binds in a specific and saturable manner to the endothelial cell surface (4). For binding to occur, glucose and the protein must have undergone a series of rearrangements to become the characteristic Maillard end product, *i.e.* AGE. Albumin glycosylated after shorter incubations with glucose, before AGEs become detectable, did not compete with ^{125}I-AGE-albumin for binding to endothelium (4). Since binding of AGE-albumin to the endothelium could be blocked by either excess unlabeled AGE-albumin or AGE-hemoglobin, but not by the same unmodified proteins (proteins incubated identically but without glucose), it was evident that the advanced glucose derivative on the protein was necessary for recognition of this cellular binding site (4).

These findings convinced us of the existence of a receptor on endothelium which mediated interaction with AGE-albumin, as well as AGE adducts of other proteins. The studies presented here indicate that two proteins, termed AGE-binding proteins, are displayed on the endothelial cell surface: an apparently novel ≈35-kDa polypeptide, and a lactoferrin-like polypeptide, and that these have a central role in the binding of AGEs. Polyclonal antibodies raised against these cell surface-binding proteins block binding of radioiodinated AGE-albumin to endothelial cells. Furthermore, polyclonal antisera prepared to each protein did not cross-react, indicating that these binding proteins are apparently distinct.

The AGE-binding proteins appear to be different from previously described albumin-binding proteins and scavenger receptors (24–28, 33, 34). Consistent with the cell binding studies described above, each of the purified AGE-binding proteins immobilized on wells of PVC plates bound ^{125}I-AGE-albumin but not the nonglycated counterpart, indicating that they are not albumin-binding proteins. Furthermore, AGE-

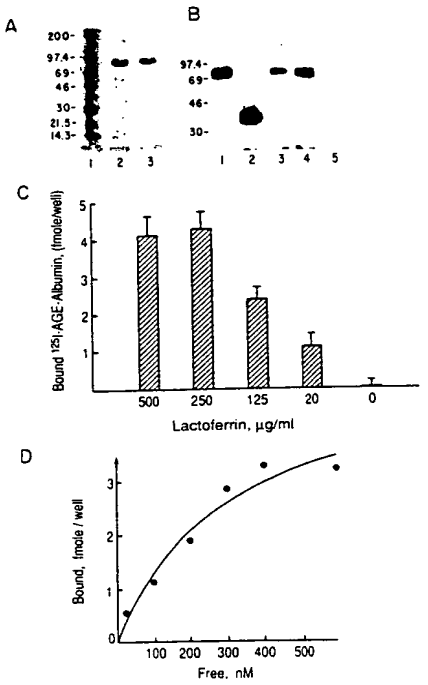

FIG. 9. **Relationship of bovine lactoferrin and the 80-kDa AGE-binding protein.** *A*, SDS-PAGE. Purified lactoferrin (*lane 2*, 1.5 μg), and purified 80-kDa AGE-binding protein (*lane 3*, 1.5 μg) were subjected to nonreduced SDS-PAGE (10–15% gradient Phast gels), and proteins were visualized by Coomassie Blue staining. Migration of standard proteins and their corresponding molecular masses (kDa) are shown in *lane 1*. *B*, immunoblotting with anti-lactoferrin antibody reacted with each of the following: *lane 1*) purified 80-kDa AGE-binding protein (4.5 μg); *lane 2*, endothelial cell extract (9 μg); *lane 3*, commercial lactoferrin (7 μg); *lane 4*, purified lactoferrin (7 μg); and *lane 5*, purified 35-kDa AGE-binding protein (4.5 μg). Samples were subjected to non-reduced SDS-PAGE (10%), followed by electroblotting onto nitrocellulose membranes. Proteins were stained with rabbit anti-bovine lactoferrin IgG (3 μg/ml) using the horseradish peroxidase method. *C*, ^{125}I-AGE albumin binding assay with purified lactoferrin immobilized on wells of PVC plates. Lactoferrin at the indicated concentration was adsorbed to PVC wells, and a binding assay with ^{125}I-AGE albumin (100 nM) alone or in the presence of a 20-fold molar excess of unlabeled material was performed. Specific binding (mean ± S.E. of quadruplicate determinations) is shown. *D*, binding of ^{125}I-AGE albumin to lactoferrin: dose-response. Lactoferrin (500 μg/ml) was adsorbed to wells of a PVC plate, and a binding assay was performed as described in the text using the indicated concentration of ^{125}I-AGE albumin alone or in the presence of a 20-fold molar excess of unlabeled material. Specific binding (the mean of quadruplicate determinations) is shown. Parameters of binding were: K_d = 268 ± 110 nM and 5.2 ± 0.1 fmol of ligand/well at saturation.

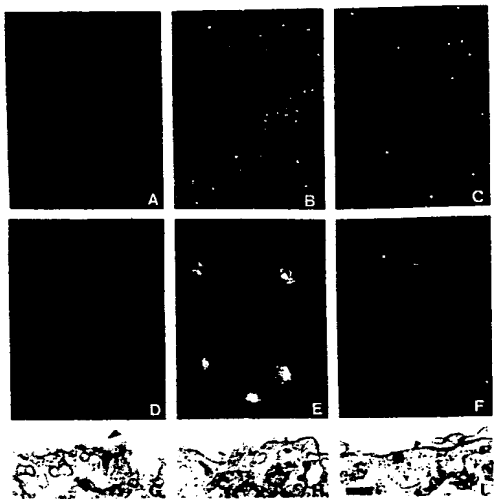

FIG. 10. **Immunofluorescence and immunoelectron microscopic study of endothelial cell monolayers stained with antibodies to purified AGE-binding proteins.** Endothelial monolayers maintained in serum-free medium for 96 h were processed for immunofluorescence as described in the text. Indirect immunofluorescence of nonpermeabilized aortic endothelial cells (*A-C*) or adrenal capillary endothelial cells (*D-F*) was then carried out using nonimmune IgG (*A* and *D*), immune IgG to the 35- (*B* and *E*) or 80-kDa (*C* and *F*) AGE-binding protein (in each case the IgG concentration was ≈15 μg/ml). Immunogold electron microscopy was performed on aortic endothelial cells by incubating cultures with anti-35-kDa AGE-binding protein IgG conjugated to 12-nm gold particles (*G*), anti-80-kDa AGE-binding protein IgG conjugated to 24-nm gold particles (*H*), or with both antibodies simultaneously (*I*). Magnification: *panels A-F*, × 650; *panels G-I*, *bar* = 100 nm.

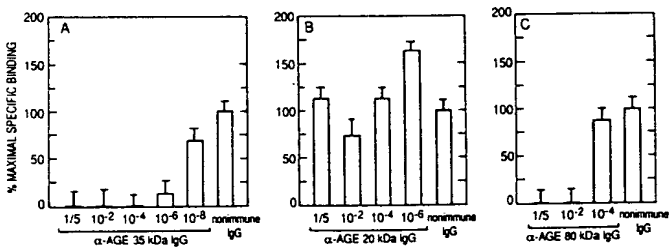

FIG. 11. **Effect of antibodies to AGE-binding proteins on the binding of ^{125}I-AGE-albumin to endothelium.** Confluent monolayers of endothelial cells were preincubated for 2 h at 4 °C with immune IgG against the purified AGE-binding proteins: anti-AGE 35-kDa IgG (*A*), anti-AGE 20-kDa IgG (*B*), and anti-AGE 80-kDa IgG (*C*). Nonimmune IgG was used as a control. Cultures were washed and then a radioligand binding assay was carried out by adding ^{125}I-AGE-albumin (100 nM) alone or in the presence of a 20-fold molar excess of unlabeled AGE-albumin. Results are shown as percent maximal specific binding (defined arbitrarily as 100% in the absence of any IgG) *versus* the dilution of IgG added (in each case the undiluted samples of immune or nonimmune IgG contained 10 μg/ml). The mean ± S.E. of triplicate determinations is shown.

modified prothrombin, but not its native form, was a competitor for the interaction of ^{125}I-AGE-albumin with the binding proteins. Thus, the AGE-binding proteins described here are distinct from previously reported albumin-binding proteins of 18 (34), 31 (34), and 60 kDa (33), which are also present on the endothelial cell surface. Furthermore, the AGE-binding proteins appear to be distinct from previously described macrophage receptors for formaldehyde-treated albumin (28). This assumption is based on mobility on SDS-PAGE (receptors for formaldehyde-treated albumin have molecular masses of 30 and 53 kDa on SDS-PAGE) and failure of formaldehyde-treated albumin to block the interaction of ^{125}I-AGE-albumin with endothelium. Similarly, the AGE-binding proteins are distinct from the scavenger receptor for acetylated LDL (26), based on comparison of the sequences and the inability of acetylated LDL to inhibit the binding of ^{125}I-AGE-BSA to endothelium. Taken together, these data are consistent with the hypothesis that the AGE-binding proteins are unique in selectively recognizing AGEs, although further studies with plasma proteins modified by other means will be required to verify this.

For preparation of AGE-binding proteins, we used an extract of bovine lung. Although the 35-kDa AGE-binding protein and material immunoreactive with the lactoferrin-like AGE-binding protein are present in cultured endothelial cells, since the lung contains many cell types, it was possible that

other cells were contributing to the pool of binding protein. In this context, previous work has shown that murine macrophages possess a binding site for AGE-albumin which is shared with FFI (2-furoyl-4-(5)-(2-furanyl)-1H-imidazole), a synthetic AGE-like compound (35). Using an 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole affinity column, a polypeptide of ≈90 kDa was isolated from membranes of transformed murine macrophages (RAW 264.7) which was postulated to be the receptor (5). This 90-kDa polypeptide may be similar/related to the lactoferrin-like polypeptide reported here, as we have identified a polypeptide immunoreactive with our anti-lactoferrin-like AGE-binding protein antibody on the human monocyte surface, and this antibody also partially blocks binding of ^{125}I-AGE-albumin to human monocytes (36). However, Yang *et al.* (37) recently published the amino-terminal sequence of the ≈90-kDa AGE-binding protein isolated from rat liver, and it does not resemble the sequence of lactoferrin or the lactoferrin-like protein we isolated from bovine lung. Further studies will be required to define the relationship between the ≈90-kDa binding protein isolated by Yang *et al.* from liver and the lactoferrin-like protein we have isolated from lung. The same applies to the relationship between our ≈35-kDa AGE-binding protein from lung and the ≈60-kDa-binding protein isolated by Yang *et al.* (37 and see accompanying article) from liver.

After soluble AGEs bind to the endothelial cell surface, they are internalized and subsequently degraded, and/or transcytosed (4). Furthermore, AGE-endothelial cell interaction can also modulate a range of endothelial cell functions (4, 6–8). In view of the presence of two AGE-binding proteins, the 35 kDa and lactoferrin-like-immunoreactive species, both of which bind AGEs individually and are involved in endothelial cell surface-AGE interaction, we speculate that they may be subunits of the cell surface-binding site. On the one hand, the presence of two AGE-binding proteins and their mode of assembly on the cell surface could facilitate their recognition of the broad spectrum of AGE derivatives which are formed (1–3). Alternatively, assembly of the binding proteins into a complex may have an important role in events subsequent to cell surface binding of the ligand, such as endocytosis, transcytosis, and activation of mechanisms coupling ligand occupancy of the receptor to signal transduction mechanisms. Our observation that antibody to either binding protein completely blocked cell surface binding of ^{125}I-AGE albumin suggests that there may be interactions between the 35-kDa and lactoferrin-like AGE-binding proteins. Consistent with this hypothesis, studies with ≈35-kDa-binding protein bound to plastic tubes demonstrated dose-dependent binding of radiodinated ≈80-kDa AGE-binding protein (38), and immunoelectron microscopic studies with gold-labeled antibodies to the two binding proteins have demonstrated their colocalization on the endothelial cell surface. Further studies will be required to confirm the existence of this complex and to determine the affinity and functional significance of the association of the 35-kDa and lactoferrin-like AGE-binding proteins on the endothelial cell surface.

To understand fully the relationship of the 80-kDa-immunoreactive AGE-binding protein to lactoferrin will also require further study, but our data thus far suggest that they are apparently identical, based on comparison of the NH_2-terminal sequence, migration on SDS-PAGE, immunoreactivity, and ^{125}I-AGE-albumin binding properties. It is known that lactoferrin exists in multiple forms and in this context the 80-kDa AGE-binding protein may prove to be an isoform (39, 40). Future studies involving cloning and expression of the 80-kDa AGE-binding protein will be required to complete its characterization and understand its relationship to lactoferrin.

Identification of the 20-kDa AGE-binding protein as high mobility 1 nuclear protein was unexpected. Presumably, this nonhistone protein (30), although able to bind AGEs under the conditions employed in our experiments, would not have access to them under physiologic conditions where the AGEs are predominately extracellular or confined within vesicular compartments within cells. Consistent with this interpretation, antibody to the 20-kDa AGE-binding protein did not inhibit cell surface binding of AGEs. However, if AGEs, which have been shown to cross-link DNA (41), could gain access to the nucleus, they could be concentrated on high mobility 1 protein and, from that initial locus, lead to perturbation of DNA structure.

The current studies have led to the identification of two endothelial cell surface-binding polypeptides which function as a receptor for AGE adducts of proteins. This work serves as a starting point for future studies of the properties of these proteins, their expression on other cell types, and their relationship to other receptors for post-translationally modified proteins. Although endothelial AGE-binding sites have been shown to bind and internalize soluble AGE-albumin (4), as would a typical scavenger receptor, the principal interaction of AGE-binding proteins with their ligand(s) may occur in the subendothelium, where AGEs have been found on long-lived basement membrane components such as collagen (1). Since AGE deposition increases with aging, and at a more rapid rate in diabetics (1), basement membrane AGEs could reach high local concentrations over time, resulting in a long term interaction with their endothelial receptors.

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